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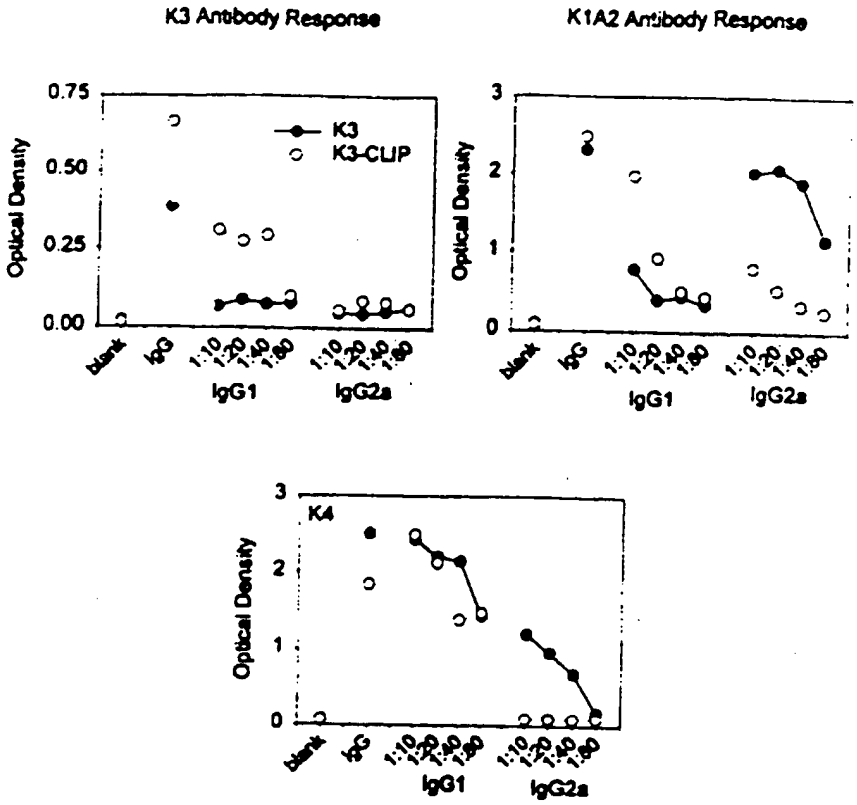
(54) **PEPTIDE CLIP IMMUNOMODULATEUR**
(54) **CLIP IMMUNOMODULATORY PEPTIDE**



(57) L'invention porte sur des compositions pharmaceutiques et immunogènes ainsi que sur des méthodes thérapeutiques, faisant appel à un peptide CLIP et utiles pour la régulation des réactions immunes chez les mammifères.

(57) The invention relates to pharmaceutical and immunogenic compositions and therapeutic methods employing a CLIP peptide and useful for regulation of immune responses in mammals.

ABSTRACT

The invention relates to pharmaceutical and immunogenic compositions and therapeutic methods employing a CLIP peptide and useful for regulation of immune responses in mammals.

CLIP IMMUNOMODULATORY PEPTIDE

Field of the Invention

This invention relates to a peptide, CLIP, which is demonstrated to be an immunodulatory peptide and can be used as a composition for the treatment of immune disease. In particular, the CLIP peptide down regulates the activity of T-cells and also influences expression and function of class II MHC molecules.

Background of the Invention

The class II major histocompatibility complex (MHC) expressed by B-cells, macrophages, dendritic cells, and thymic epithelial cells is a heterodimeric molecule with two closely related chains. These two chains, the α and β, are involved in binding peptides by virtue of highly polymorphic residues found at the N-terminal domains (1). The class II MHC functions by binding self-peptides in the major groove and guiding the selection of CD4+ cells in order to initiate immune responses.

After synthesis, the class II MHC molecule associates with the invariant chain (Ii) which serves to prevent premature binding (2). The Ii undergoes degradation which leaves only the peptide portion (aa residues 85-101), also called CLIP, in contact with the a/b complex (3,4). The CLIP fragment has also been demonstrated to prevent peptide binding (5,6) and is, therefore, assumed to be tightly bound to the binding groove of the MHC. It is necessary to remove CLIP from the binding groove in order for peptide antigens to bind to the MHC. The removal of CLIP from the binding groove is a task facilitated by HLA-DM.

HLA-DM plays a vital role in the removal of CLIP and allowing antigens to bind to the core binding groove of the class II MHC. In the absence of HLA-DM, CLIP prevents the binding of antigens in the binding groove and their subsequent presentation on the cell surface (7). We now show that increasing the amount of CLIP saturates the endosome and inhibits the ability of antigen to bind. We

also now report that altering the expression of CLIP inside the cell will clearly affect the ability of peptides to bind the MHC. We have now more clearly defined the function of CLIP as a regulator of the density of peptides being presented at the surface of the APC (antigen presenting cells). We also demonstrate that CLIP levels modulate the activity of CD4+ T-cells and the subsets of T-cells generated. We therefore propose that CLIP can be used as an immunomodulatory protein in general and more specifically it can be used to treat immune disorders involving increased T-cell activation and inappropriate sensitization of the immunological defences of the body resulting in self-destruction and typically known as autoimmune disorders. The peptide can also be used for transplantation and for combatting infection.

Summary of the Invention

The present inventors have now characterized the function of the CLIP peptide and its effects on immunological function. The identification of the role of the CLIP peptide permits the development of therapeutic strategies in order to combat autoimmune disorders as well as for use in tissue transplantation and for treating infection.

The present invention relates to an isolated CLIP peptide which can be used and administered as a therapeutic composition for the treatment of disorders involving the immune system.

In accordance with one embodiment the present invention is a therapeutic composition containing the CLIP peptide, or an active analogue or fragment thereof in a composition suitable for oral or parenteral delivery for treatment of immune disorders. The composition may be delivered in a suitable vehicle, microencapsulated or provided in a liposome and targeted to a specific site with or without a carrier. The composition may also be

administered in order to minimize or prevent tissue graft rejection after transplantation as well as for treating those infections where the immune response is overcompensating and thus creating more tissue damage.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

The invention will now be described in relation to the drawings in which:

Figure 1 shows the amino acid sequence of the CLIP peptide from humans (SEQ ID NO:2) and mice (SEQ ID NO:1).

Figure 2 shows the effect of mouse CLIP peptide on the expression of cell surface class II MHC in TA3 cells. A) Staining of TA3 cells with mAb MK-D6 (anti-I-Ad) or 10-2.16 (anti-I-Alc) after 24 h of incubation with various concentrations of CLIP. A dose-dependent decrease in surface class II MHC is apparent in both instances. B) Staining as described above for I-Ad after incubating TA3 cells with HEL or OVA for 24 h. No effect on I-Ad surface expression is seen with HEL, which is predominantly I-Ak restricted, while OVA significantly up-regulated I-Ad expression. In each case, background staining was determined using only secondary Ab (FITC-goat anti-mouse IgG).

Figure 3 shows the effect of CLIP peptide incubation with TA3 cells on the intracellular formation of stable, SDS-resistant, compact MHC class II a/b chain heterodimers. Cells were incubated for 24 h in the presence of various

concentrations of CLIP. Cell lysates were prepared and loaded onto a 10% SDS-PAGE gel with or without heating (95oC, 5 min) and analyzed by Western blotting using the I-Ad binding mAb, MK-D6. The gels were analyzed by densitometry, and the data are presented as the percent decrease in the compact state of I-Ad (non-boiled samples) with respect to total intracellular I-Ad, shown as a function of the dose of CLIP. Note that at higher CLIP concentrations (200 µg/ml), saturation of the system is reached.

Figure 4 shows the binding of immunogenic peptides and CLIP to surface class II MHC molecules. A) CLIP-incubated cells (left) fluoresce weakly, whereas OVA-incubated cells (right) fluoresce strongly over time indicating binding. B) Time course of the binding of FITC-CLIP and FITC-OVA from A), demonstrating a leveling off or plateauing of peptide surface presentation over time in the CLIP group, but not in the OVA peptide group. TA3 cells were incubated in the presence of FITC-OVA-(323-339) or FITC-CLIP at 100 µg/ml. After various times, cells were washed and analyzed by cells surface FACS. FITC-peptides were made as described in the examples such that only one FITC group was attached at the N-terminal of the peptide molecule.

Figure 5 shows the effect of immunization with CLIP peptide (85-101) on the T cell response. A) Proliferation of BALB/c-derived lymph node cells from mice immunized as described and challenged according to the abscissa. B) Proliferation of C3H-derived lymph node cells as described above without the K1A2 group. C) Proliferation of BALB/c-derived lymph node cells after enriching for APCs and mixing with nylon wool purified T cells as described. D) Dose-response curves of the down-regulation of PPD-specific T cell responses by CLIP. Shown is the proliferation of BALB/c-derived cells from mice immunized as described and challenged by using different concentrations of PPD (micrograms per millileter) according to the abscissa. E)

Down-regulation of OVA-specific T cell responses by CLIP. For the above experiments, mice were immunized with Ag in CFA or IFA using 100 µg/footpad of CLIP and 50 µg/footpad of other Ags. On day 10, T cell proliferation was measured to determine the recall response to specific Ags. Assays were performed in the presence of priming Ag in culture medium using 100 µg/ml of CLIP, 50 µg/ml of other Ags, and 40 µg/ml of PPD unless otherwise indicated.

Figure 6 shows the effect of immunization with CLIP peptide (85-101) on MHC class II on lymph node APCs. A) Staining with RA3.3A1 demonstrates that immunized CLIP has no significant effect on B220 (CD45) surface expression, whereas in B) staining with MK-D6 shows substantial down-regulation of surface I-Ad. In A) and B), BALB/c mice were immunized in the hind footpads with CFA and CLIP (100 µg/footpad) using CFA and saline as a control. On day 10, lymph node cells were harvested, stained, and analyzed by FACS as described in the examples.

Figure 7 shows the effect of immunization with CLIP on the proliferative response to peptide antigens.

Figure 8 shows the effect of immunization with CLIP on the generation of TH1 and TH2 cells. A) IL-4 is assayed as a result of CLIP immunization. B) IFN-γ is assayed as a result of CLIP immunization.

Figure 9 shows the effect of CLIP on the antibody response to K3, K4 and K1A2. BALB/c mice were immunized with 50 µg of CLIP along with either K3, K4 or K1A2 in IFA and compared to BALB/c mice immunized without CLIP. After two weeks a similar injection was given i.p. in IFA serum was collected and levels of IgG1 and IgG2a antibodies were determined as described in the examples.

Figure 10 shows the effect of CLIP on the proliferative response of whole ovalbumin. Mice were immunized with either ovalalbumin or ovalalbumin-CLIP with ovalalbumin-K1A2 as the control. Lymph nodes were

harvested after 10 days, cells were cultured with 50 µg/ml of peptide and proliferation was assayed as described in the examples.

Figure 11 shows the effect of CLIP on the generation of TH1/TH2 cells in response to ovalalbumin. T-cells were incubated with ovalalbumin and the supernatants assayed for IL-4 and IFN-γ.

Detailed Description of the Preferred Embodiments

The present invention relates to the use of a CLIP peptide as an immunomodulatory protein which can be isolated and used to treat immune disorders involving increased T-cell activation, such as autoimmune disorders as well as for tissue transplantation and for combatting infection.

"CLIP peptide" means the portion of the MHC class II invariant chain protein which occupies the MHC II groove and includes human invariant chain amino acids 81 to 104 and homologous stretches of the amino acid sequence of the MHC class II invariant chain protein of other species.

The CLIP peptide can be administered to decrease antigen presentation to the class II MHC. Furthermore, CLIP also is shown to increase the production of IL-4 and decrease the production of IFN-γ. The CLIP peptide is also useful for shifting the immune T cell response from a TH1 inflammatory response to a TH2 protective response which further supports its use as a therapeutic agent for the treatment of immune disorders.

Also included within the scope of the invention is the use of active fragments or analogues of a CLIP protein and of polypeptides which include the amino acid sequence of a CLIP peptide or fragments thereof.

By "activity" is meant any function regulated or modulated by CLIP peptide.

In preferred embodiments, the activity is down-regulation of the surface expression of MHC class II

molecules on the surface of antigen presenting cells and/or the resulting reduction or prevention of a T cell response to an antigen.

An "active fragment" or "active analogue" of MHC class II-associated in variant chain protein or of CLIP peptide is a fragment or analogue which retains a function regulated or modulated by CLIP peptide.

Active analogues of human CLIP peptide include CLIP peptides from other species. For example, murine CLIP (Figure 1) is active in human cell systems.

One of ordinary skill in the art can readily screen fragments or analogues of CLIP for activity by the assays described herein. Such assays may include adding fragments or derived analogues of CLIP to an appropriate cell culture such as BALB/c cells which express surface I-A^d and I-A^k. Cells can then be appropriately stained to quantitate surface expression of I-A^d and I-A^k by FACS analysis in order to determine any down-regulation of I-A^d or I-A^k. The ability of CLIP fragments and/or analogues to affect cytokine production such as IL-2 or IFN-γ can also be assayed using lymph node cells cultured from mice immunized with the desired fragments and/or analogues. Supernatants from such cultures are then collected and assayed for different cytokine concentrations.

It is also possible to assay for the ability of the CLIP fragments and analogues to bind to cell surface class II MHC using FACS analysis. Finally, T-cell responses in lymph node cells from immunized BALB/c mice can be assayed as a result of using a CLIP fragment or analogue for immunization.

All of the assay systems are described herein in the examples and provide a means for identifying those fragments and analogues of CLIP which can be used as a pharmaceutical therapeutic and immunogenic composition.

The Role of CLIP on Modulating Antigen Presentation and Class II Expression

Considerable evidence has recently been obtained supporting the idea that CLIP (4,5') binds in the Ag binding groove of class II molecules (7, 14, 16, 17'). No functional evidence exists for the *in vivo* role of CLIP despite the speculation that CLIP could accumulate on class II molecules in cells that lack HLA-DM proteins and, in turn, block the binding of antigenic peptide to class II MHC molecules (18'). It is now discovered that the saturation of the endosomal compartment with exogenously added CLIP would result in reduced surface expression of class II molecules and would inhibit Ag presentation to CD4+ T cells. This provides direct evidence for its functional role and suggests novel ways to modify Ag presentation by class II MHC molecules. More broadly stated, the present invention now shows the immunologic role of the CLIP peptide.

The CLIP peptide of the MHC class II pathway is able to impede efficient Ag presentation by APCs *in vivo*. *In vitro*, TA3-B cell hybridomas incubated with exogenous CLIP internalize the peptide where it has a dramatic effect on achieving compact, stable, class II heterodimers and this is reflected as a decrease in surface class II MHC. *In vivo*, administration of CLIP indirectly impedes T cell priming, presumably by reducing the quantity of other exogenous peptides available for presentation by APCs.

For the *in vitro* studies, a B cell hybridoma, TA3 was used which is a good APC (12'). Using the I-A^d-restricted Ag, OVA we have found that the addition of CLIP peptide decreased the surface expression of I-A^d and I-A^k. This is consistent with the observation that CLIP has >1000-fold higher affinity for I-Ad than I-A^k (14'). The observed down-regulation of surface class II MHC could be the result of altered conformation-dependent class II epitopes, as have been previously reported in HLA-DM mutant cell lines

(18,19') rather than of CLIP per se. These mutant cell lines also have a large number of their class II molecules occupied by CLIP (18'). Significantly, however, this phenomenon is only seen in cells with defective or deleted DM proteins. We have shown that H-2M is probably not defective in the TA3 cell line in two significant ways. TA3 subclones, although differing in constitutive levels of surface I-A^d, retain relatively constant levels of H-2 M. Also, defects in the HLA-DM protein in cell lines have been shown to be correlated with an inability to efficiently present native protein (19,20'). When comparing a high I-A^d-expressing subclone of TA3 with a low I-Ad-expressing subclone, we found no significant difference in the ability to present either native protein or peptides (data not shown).

It is unlikely that the CLIP-class II complexes are recognized inefficiently by the Abs used because the MHC down-regulation is found using Abs that target two different MHC haplotypes (i.e. I-Ad and I-A^k) and the Western blot analysis revealed that MK-D6 Ab binds to stable as well as unstable heterodimers which substantiates that MK-DK does not bind in a conformation-specific manner. Also, this data is consistent with data showing that both 10-2.16 (anti-I-Ab^k) and MK-DK (anti-I-Ab^d). Abs efficiently immunoprecipitate compact and SDS-unstable dimers (15') and FACS analysis performed by others (21') has shown that while other Abs are sensitive to conformational changes induced by the presence or the absence of Ii, MK-D6 remains insensitive, suggesting that MK-D6 binds independently of conformations induced by the peptides in the class II binding site.

The *in vivo* CLIP data illustrated a similar effect. Mice immunized with CLIP had a reduction in the expression of class II molecules on the APCs. These results support the idea that exogenously added CLIP is taken up by the APCs and this results in the inhibition of antigenic

peptides loading onto the MHC class II molecules in the endosomal compartment. It is possible that the observed reduction in class II MHC on BALB/c and C3H cells was the result of a lack of T cell activation in the presence of CLIP rather than a direct effect of CLIP blocking. Decreased T cell activation has been observed in animals immunized with CLIP and this may in turn, result in decreased levels of MHC-up-regulating cytokines (22'). Although CLIP could apparently inhibit Ag presentation *in vivo*, this effect was not apparent when cells were subsequently challenged in vitro. It is possible that T cell priming is more sensitive to adequate ligand density via MHC on APCs. Should this not occur, T cells may be tolerized rather than primed or activated. Considering this, if APCs are presenting fewer ligands of peptide Ag due to the presence of CLIP, a decrease *in vivo* priming events should be seen. This would be consistent with studies in which mice expressing low levels of surface class II MHC show drastically impeded T cell responses (23').

The observation that immunogens are capable of up-regulating class II MHC is further evidence that exogenously added Ags influence class II surface expression (12). It is likely that immunogenic peptides stabilize class II heterodimers, which leads to long lived surface complexes.

Because of the apparent correlation between dose and time of exposure of cells to CLIP, it is presumable that CLIP is exerting a role in down-regulating the surface expression of class II MHC. Further support for this comes from Western blot analysis that demonstrates an increase in the intracellular floppy state of MHC class II molecules compared with that of non-CLIP incubated cells. This is significant because it has been reported that MHC molecules bound strictly to CLIP peptide do not achieve the compact SDS-stable state (7').

Defective Ag presentation has been shown to be linked to the inability of HLA-DM molecules (the human counterpart to murine H-2M molecules) to remove invariant peptides and that once functional DM molecules are transfected to these cells, normal Ag presentation resumes (18'). The observations may be linked to the function of the H-2M accessory protein, which is believed to play a vital role in the endocytic pathway (6, 24') similar to that of HLA-DM. By incubating APCs with CLIP, we have in effect artificially enhanced its presence in the endocytic pathway. Here, we believe that the compartment of peptide loading (CPL or CIIV) is saturated with CLIP and H-2M is unable to perform adequately in removing the peptide occurring as a result of self-processing activity. Because of this, CLIP remains bound with MHC molecules and prevents them from having their binding sites occupied by exogenous peptides. For this to be true, it must be demonstrated that exogenous CLIP is in fact being internalized. The preliminary confocal microscopy results (data not shown) revealed that CLIP is in fact internalized over time and colocalizes intracellularly with a vesicle that is rich in class II MHC and is proximal to the cell surface. Also, incubating TA3 cells with FITC-CLIP or FITC-OVA-(323-339) demonstrated that OVA peptide binds strongly and immediately to surface class II molecules, whereas CLIP does not. Moreover, these observations do not appear to be brought about by the ability of CLIP to down-regulate class II MHC, since down-regulation is not apparent until after 4h of culture. These observations lead to a strong indication that exogenous CLIP is being internalized along the endocytic pathway. It has also been shown that class II peptide complexes once formed are virtually irreversible (25'). Given this, it is difficult to rationalize exogenous CLIP exerting its effect in any other way than by interfering at the stage where APC is endogenously loading the processed Ag onto class II molecules, since the

mycobacterial components in CFA require processing before presentation. Therefore, other than associating with class II MHC, as is the case with OVA-(323-339) and other immunogenic peptides, CLIP is most likely interfering endogenously, perhaps with H-2M, which prevents the export of functional class II-immunogenic peptide complexes to the cell surface.

The functional effect of CLIP on T cell responses is further supported by its role in Ag presentation. We found that CLIP inhibited the T cell response when injected simultaneously with Ag. This suggests that either CLIP prevents presentation of Ag to T cells or blocks T cell function. Cell mixing experiments supported the functional role of CLIP in blocking Ag presentation by the APCs. When T cells and APCs from mice immunized with CLIP plus CFA or with CFA alone were mixed, we found that APCs from the CLIP/CFRA-immunized mice inhibited the presentation of PPD to T cells from the CFA alone group. Mixing T cells from CLIP-immunized mice with APCs from saline-immunized mice revealed a similarly reduced in vitro T cell response, suggesting that T cells were inefficiently primed in vivo due to inefficient presentation and low class II MHC on the APCs (23').

The data in Figure 5A, where a different peptide of similar size to CLIP is used in its place for immunization, shows that in vivo T cell priming is impaired not as the result of direct competition between CLIP and other immunized Ag but rather as a result of the interaction between CLIP and the endocytic pathway.

These results support the idea that the level of intracellular CLIP is normally in an equilibrium that affords the cell a balance between efficient peptide presentation and maintaining the pool of class II MHC to present antigenic peptide. When this equilibrium is altered by exogenous CLIP, efficient peptide presentation by MHC class II molecules is drastically impeded, which in

turn, down-regulates Ag-specific T cell responses. Saturating quantities of CLIP peptide prevents adequate peptide loading of exogenous antigenic peptide, which in turn impedes the formation of the compact state of class II heterodimers. The ultimate effect of reducing the number of cell surface-stable class II heterodimers is a reduction in the efficiency of APC Ag presentation. In support of these results, studies in HLA-DMB mutants have demonstrated that the inability to remove Ii peptides prevents the formation of stable HLA-DR molecules (18'). These data demonstrate the ability of CLIP, administered exogenously, to down-regulate the immune response by blocking efficient Ag presentation to T cells. Because of its ability to bind to MHC class II α/β heterodimers in the peptide loading compartment, exogenously added CLIP inhibits the principal function of the H-2M molecule in situ. These studies confirm the intracellular role of CLIP in Ag presentation.

The Role of Clip On the Immune Response Induced on TH1 and TH2 by Different Peptide Antigens

Also investigated was the effect of CLIP on the immune response induced by different peptide antigens with different affinities for MHC. The peptide K3 has a low affinity and has been shown to induce a TH2 type of response while K1A2 with a high affinity has been shown to induce a TH1 type of response. The peptide K4 has an intermediate affinity and induces both TH1 and TH2 types of responses (8'). We now show that saturating the endosomal compartment with exogenously added CLIP would down regulate the antigen presentation which in turn may affect the cytokine profile and perhaps completely alter the immune response to a specific antigen.

When immunizing CLIP with the three peptide antigens a variation in response to the peptides K3, K4 and K1A2 was noted. The peptide K1A2 induced a strong proliferative response whereas the peptide K3 generated only a weak

proliferative response. The peptide K4, on the other hand, produced a moderate response. This confirms earlier work done with these peptides (8'). This variation in response is a consequence of the fact that the peptides, though cross reactive, have minor differences in their amino acid structure at key residues resulting in different affinities for I-A^d.

The inventor has shown that when K3, K4 and K1A2 were injected along with CLIP, a significant decrease in the proliferative response towards each of the peptides was observed. Also observed was that K3 and K4 consistently demonstrated a significant drop in response when immunized with CLIP whereas K1A2 experienced a reduction ranging from minimal to significant (data not shown). It is reasonable to assume that CLIP, like the peptide antigens K3, K4 and K1A2, becomes internalized and localized in the endosome. As both CLIP and the peptides are able to bind to class II MHC, they compete with each other for the privilege. As a result, antigens experience a decreased rate of binding to the MHC and a lower level of presentation. This results in a lower ligand density and is, therefore, responsible for the observed weaker immune response. The peptide K1A2, due to its high affinity, was able to compete more effectively with CLIP than the other peptides and therefore did not experience as great a decline in the level of peptide binding or presentation and in turn, did not show as noticeable a decrease in response.

The degree to which CLIP is able to inhibit antigen binding and subsequent antigen presentation is related to the affinity of the antigen for I-A^d. High affinity antigens appear to be effective at competing with CLIP and therefore are still able to mount a significant response. Lower affinity antigens appear to be less capable of doing this and a greater drop in proliferative response is observed.

It has been previously demonstrated that ligand density, along with the type of APC and particular epitope presented, is an important factor in determining whether a Th1-like or a Th2-like response is generated (8', 17'). Important for our study, it has been shown that a high ligand density is responsible for generating Th1 cell responses, where as Th2 cell responses result from a low ligand density. If, as evidence indicates, CLIP is truly altering the expression of peptides at the surface of APC's and as a result is affecting T-cell activation and proliferation then it would be expected that CLIP would also affect cytokine profiles. This is, indeed, what we found.

The notion that CLIP down regulates MHC expression is supported by its ability to alter cytokine secretion. We observed that CLIP causes greater amounts of IL-4 to be released in mice immunized with either K3, K4, or K1A2. This increase in IL-4 would correspond to an increase in the relative amount of Th2 cells generated by the immune response. Also observed, was the fact that CLIP induced a decrease in the amount of IFNγ secreted, indicating there was a drop proportion of Th1 cells. All in all, CLIP demonstrated an ability to shift the T-helper cell response away from a Th1-like and towards a Th2-like.

The results from the antibody studies also reveal a shift from a Th1 towards a Th2 response. Immunization with CLIP was observed to change the proportions of IgG1 and IgG2a antibodies. As a result of CLIP, antibody responses to each peptide showed a significant increase in the proportions of IgG1 antibodies, as well as a decrease in the proportion of IgGa antibodies. As IgG1 antibodies are associated with a Th2 response and IgG2a antibodies with a Th1 response (13'), this shows again that there is a switch from Th1 to Th2 cells.

The change in T-helper cell subsets is an important aspect of a treatment with a CLIP-containing immunogenic

composition. It alters the very nature of the immune response as it does not stimulate antibody production because the peptide is non-antigenic. Rather than being inclined towards an inflammatory response (Th1), an immune reaction can be directed towards a more protective response (Th2).

Under normal situations, immune systems rarely see peptide antigens as described above but rather encounter protein antigens. Protein antigens require an important additional step, in that it is necessary for them to undergo processing before binding to class II MHC. The inventor has shown that CLIP had a similar effect on the ovalbumin as it did on the peptides K3, K4, and K1A2. It caused a decrease in proliferative response. It also induced an increase in the levels of Il-4 secretion and a correlating decrease in IFN-γ levels. Apparently, there is no difference in the effect of CLIP on peptides or protein.

The CLIP peptide has been demonstrated to be effective at transforming the immune response. It is able to down regulate the strength of the proliferative response without eliminating it. CLIP is also able to alter the nature of the T-helper subset populations and as a result change the way in which the immune system responds to antigens. The Th2 subset is important for the generation of humoral immune responses whereas the Th1 subset plays a critical role in generating delayed-type hypersensitivity immune responses. The Th1 subsets are often found to also be involved with autoimmune conditions. They play an important role in causing inflammatory disorders as well. A technique such as using CLIP, provides a means by which to manipulate Th1 subsets could prove useful in controlling autoimmune disorders. CLIP may be an effective tool to modulate responses and shift them away from a harmful, disease causing Th1 immune response towards a Th2 response which often associated with protection.

Peptides

CLIP peptides or fragments thereof may be prepared by any suitable peptide synthetic method.

Chemical synthesis may be employed, for example standard solid phase peptide synthetic techniques may be used. In standard solid phase peptide synthesis, peptides of varying length can be prepared using commercially available equipment. This equipment can be obtained from Applied Biosystems (Foster City, CA.). The reaction conditions in peptide synthesis are optimized to prevent isomerization of stereochemical centres, to prevent side reactions and to obtain high yields. The peptides are synthesized using standard automated protocols, using t-butoxycarbonyl-alpha-amino acids, and following the manufacturer's instructions for blocking interfering groups, protecting the amino acid to be reacted, coupling, deprotecting and capping of unreacted residues. The solid support is generally based on a polystyrene resin, the resin acting both as a support for the growing peptide chain, and as a protective group for the carboxy terminus. Cleavage from the resin yields the free carboxylic acid. Peptides are purified by HPLC techniques, for example on a preparative C18 reverse phase column, using acetonitrile gradients in 0.1% trifluoroacetic acid, followed by vacuum drying.

CLIP peptides may also be produced by recombinant synthesis. A DNA sequence encoding the desired peptide is prepared, for example by cloning the required fragment from the DNA sequence encoding the complete invariant chain protein, obtainable from genomic DNA or from commercially available genomic or cDNA libraries, and subcloning into an expression plasmid DNA. Suitable mammalian expression plasmids include pRC/CMV from Invitrogen Inc. The gene construct is expressed in a suitable cell line, such as a Cos or CHO cell line and the expressed peptide is extracted and purified by conventional methods. Suitable methods for recombinant synthesis of peptides are described in

"Molecular Cloning" (Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989).

Analogues of CLIP peptides may be prepared by similar synthetic methods. The term "analogue" extends to any functional and/or chemical equivalent of a CLIP peptide and includes peptides having one or more conservative amino acid substitutions, peptides incorporating unnatural amino acids and peptides having modified side chains.

Examples of side chain modifications contemplated by the present invention include modification of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acetylation with acetic anhydride; carbamylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulfonic acid (TNBS); alkylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2, 3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via -acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulfhydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulfonic acid, phenylmercury chloride, 2-chloromercuric-4-nitrophenol and

other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tyrosine residues may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodacetic acid derivatives of N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid-, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers or amino acids.

Examples of conservative amino acid substitutions are substitutions within the following five groups of amino acids (amino acids are identified by the conventional single letter code): Group 1: F Y W; Group 2: V L I; Group 3: H K R; Group 4: M S T P A G; Group 5: D E.

Also included in the present invention are CLIP analogues such as those described in Gautam et al., (1995) and Jensen (1996), the contents of which are incorporated herein by reference. These studies examined the effect of amino acid substitution on CLIP binding to the MHC groove and described analogues retaining CLIP binding activity. It is predicted that these analogues will be effective in the methods of the present invention.

The discovery that the CLIP peptide is internalized and decreases surface expression of class II MHC molecules as well as inhibits antigen presentation to T cells and decreases T cell function, clearly points to a therapeutic

role for this peptide. In addition, the finding that CLIP alters cytokine production and alters TH1 and TH2 responses clearly indicates its role as a therapeutic composition for those conditions involving inappropriate immune responses and in particular autoimmune disorders such as lupus, diabetes, rheumatoid arthritis just to name a few. Autoimmune disorders result from a failure of the immune system to discriminate between foreign antigen and self antigens and can result in death.

In accordance with one embodiment of the invention, CLIP peptide or an active fragment or analogue thereof is administered to a mammal in need of treatment as a pharmaceutical composition comprising a solution of the peptide, and optionally including a pharmaceutically acceptable carrier.

A pharmaceutical composition comprising a solution of a CLIP peptide acts as an anti-inflammatory and leads to down-regulation of the expression of MHC class II molecules.

The composition may be administered in a safe and effective amount to any living organism including humans and animals. By safe and effective as used herein is meant providing sufficient potency in order to decrease, prevent, ameliorate or treat the immune condition affecting a subject while avoiding serious side effects. Such a safe and effective amount will vary depending on the age of the subject, the physical condition of the subject being treated, the severity of the immune condition, the duration of treatment and the nature of any concurrent therapy. Administration of a therapeutically active amount of the pharmaceutical composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. This may also vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the CLIP peptide to elicit a desired response in the subject.

Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

By pharmaceutical carrier as used herein is meant one or more biologically compatible solid or liquid delivery systems. By biologically compatible as used herein is meant that the components of the composition are capable of being commingled, without interacting in a manner that would substantially decrease the pharmaceutical efficacy of the total CLIP composition which includes the liposome delivery system under ordinary use. Some examples of compatible materials useful as pharmaceutical carriers are sugars, starches, cellulose and its derivatives, powdered tragacanth, malt, gelatin, collagen, talc, stearic acids, magnesium stearate, calcium sulfate, vegetable oils, polyols, agar, alginic acids, pyrogen-free water, isotonic saline, phosphate buffer, and other suitable non-toxic substances used in pharmaceutical formulations. Other excipients such as wetting agents and lubricants, tableting agents, stabilizers, anti-oxidants and preservatives are also contemplated.

The peptide solution may be administered therapeutically by injection or by oral, nasal, buccal, rectal, vaginal, transdermal or ocular routes in a variety of formulations, as is known to those in the art.

For oral administration, various techniques can be used to improve peptide stability, based for example on chemical modification, formulation and use of protease inhibitors. Stability can be improved if synthetic amino acids are used, such as peptoids or betidamino acids, or if metabolically stable analogues are prepared.

Formulation may be, for example, in liposomes for improved stability. Oral administration of peptides may be accompanied by protease inhibitors such as aprotinin,

soybean trypsin inhibitor or FK-448, to provide protection for the peptide. Suitable methods for preparation of oral formulations of peptide drugs have been described, for example, by Saffran et al., (1979) (use of trasylol protease inhibitor); Lundin et al. (1986) and Vilhardt et al., (1986).

The composition containing the CLIP peptide of the present invention can also be administered in an solution or emulsion contained within phospholipid vesicles called liposomes. The liposomes may be unilamellar or multilamellar and are formed of constituents selected from phosphatidylcholine, dipalmitoylphosphatidylcholine, cholesterol, phsphatidylethanolamine, phsophatidylserine, demyristoylphosphatidylcholine and combinations thereof. The multilamellar liposomes comprise multilamellar vesicles of similar composition to unilamellar vesicles, but are prepared so as to result in a plurality of compartments in which the CLIP containing solution or emulsion is entrapped. Additionally, other adjuvants and modifiers may be included in the liposomal formulation such as polyethyleneglycol, other peptides or other materials.

The liposomes containing the CLIP composition may also have modifications such as having antibodies immobilized onto the surface of the liposome in order to target their delivery.

Due to the high surface area and extensive vascular network, the nasal cavity provides a good site for absorption of both lipophilic and hydrophilic drugs, especially when coadministered with absorption enhancers. The nasal absorption of peptide-based drugs can be improved by using aminoboronic acid derivatives, amastatin, and other enzyme inhibitors as absorption enhancers and by using surfactants such as sodium glycolate, as described in Amidon et al., (1994).

The transdermal route provides good control of delivery and maintenance of the therapeutic level of drug.

over a prolonged period of time. A means of increasing skin permeability is desirable, to provide for systemic access of peptides. For example, iontophoresis can be used as an active driving force for charged peptides or chemical enhancers such as the nonionic surfactant n-decylmethyl sulfoxide (NDMS) can be used.

Transdermal delivery of peptides is described in Amidon et al. (1994) and Choi et al. (1990).

Peptides may also be conjugated with water soluble polymers such as polyethylene glycol, dextran or albumin or incorporated into drug delivery systems such as polymeric matrices to increase plasma half-life.

More generally, formulations suitable for particular modes of administration of peptides are described, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company (Easton, PA.)

Immunogenic Compositions

In accordance with a further embodiment of the invention, CLIP peptide or an active fragment or analogue thereof is administered as an immunogenic composition to a mammal in need of treatment.

Immunogenic compositions of CLIP peptide do not stimulate any antibody response (B.J. Rider et al. (1996), *Molec. Immunol.*, v. 33, p. 625) but do modulate the response of the T cell arm of the immune system to an antigen. In addition to down-regulating surface expression of MHC class II molecules, immunogenic compositions of CLIP cause a shift in the ratio of TH1:TH2 helper cells.

Immunogenic compositions of CLIP or an active fragment or analogue thereof may be prepared by combining the selected peptide with a suitable adjuvant.

Adjuvants may be employed which not only enhance but selectively modulate the type of immune response to the administered peptide; for example monophosphoryl lipid A (MPL) favours a TH1 type response, while QS21 (Cambridge Biotech) favours a cytotoxic T cell response.

Aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are adjuvants commonly used in human and veterinary vaccines. Olive oil emulsions or other human-approved emulsifying agents may also be used.

An adjuvant should be non-toxic, capable of stimulating a sustained immune response and compatible with the peptide.

Immunogenic compositions containing proteins or peptides are generally well known in the art, as exemplified by U.S. Patents 4,601,903; 4,599,231; 4,599,230; and 4,596,792; all of which references are incorporated herein by reference. Immunogenic compositions may be prepared as injectables, as liquid solutions or as emulsions. CLIP peptides or analogues or fragments thereof may be mixed with pharmaceutically acceptable excipients which are compatible with the peptides, fragments or analogues. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof.

The immunogenic compositions of the invention may further contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness of the vaccines.

Immunogenic compositions may be administered parenterally, or by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. The oral, nasal, vaginal, gastrointestinal, respiratory or other mucosal route of vaccine administration may be preferred to combat infections which take place at mucosal surfaces, for example in the respiratory, digestive or urogenital tracts. Nasal immunization has been shown to be efficacious in generating both respiratory tract mucosal immunity and systemic immunity. Inhalation of an aerosol formulation may also be used to combat lung or respiratory tract infections.

Delivery systems for mucosal immunization include lipid vesicles, biodegradable microcapsules, attenuated bacteria, live viral vectors and bacterial toxins or subunits thereof.

For examples, cholera toxin B subunit may be conjugated to an antigen for improved mucosal immunization.

Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed excipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. Immunogenic compositions may take the form of solutions, aerosols, suspensions, tablets, pills, capsules, sustained release formulations or powders and may comprise 10-95% of peptide 15 or peptide 42 or an analogue or fragment of one of these peptides.

The immunogenic compositions are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity of immunogenic composition to be administered depends on the subject to be treated, including, for example, the weight of the subject and the capacity of the subject's immune system to produce a cell-mediated immune response. The dosage may also depend on the route of administration. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the peptides, analogues or fragments thereof.

Varying concentrations of CLIP may be used depending on the affinity of the antigen involved.

Nucleic acid molecules encoding CLIP peptide or a fragment or an analogue thereof may also be used for immunization. For example, DNA in a plasmid vector may be administered directly, in saline, by injection, preferably by intramuscular injection, for genetic immunization. It is believed that the DNA is expressed *in vivo* to give the encoded peptide antigen which stimulates an immune response. DNA may also be administered by constructing a live vector such as *Salmonella*, BCG, adenovirus, poxvirus, vaccinia or poliovirus including the DNA. Some live vectors that have been used to carry heterologous antigens to the immune system are discussed

in, for example, O'Hagan (31). Processes for the direct injection of DNA into subjects for genetic immunization are described in, for example, Ulmer et al., (32).

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of molecular genetics, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

EXAMPLES

Example 1

Materials and Methods

Mice

Female BALB/c (I-A^d) and C3H/HEJ (I-A^k) mice, 7 to 12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens

CFA, IFA, OVA, hen egg lyzozyme (HEL), and purified protein derivative of *Mycobacterium tuberculosis* (PPD) used for immunizations and in vitro were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse CLIP-(85-101), K1A2, and OVA-(323-339) peptides were synthesized in this laboratory using an Applied Biosystem 431A peptide synthesizer (Foster City, CA), as previously described (10, 11), according to the sequence KPVSQMRMATPLLMRPM (SEQ ID NO:1) (CLIP), EYKEYAAYAEYAEYA (SEQ ID NO:3) (K1A2), or ISQAVHAAHAEINEAGR (SEQ ID NO:4) (OVA). K1A2 has been described previously (10) and shows an IC_{50} value of 0.27 μM for binding to purified class II I-A^k molecules (11). Peptides were purified using reverse phase HPLC on a C_{18} analytical column (YMC, Kuse-gun, Kyoto, Japan), using a linear gradient of water-acetonitrile (1.37% acetonitrile/min). FITC-CLIP-(85-101) and FITC-OVA-(323-339) were prepared using an *N*-hydroxysuccinamide-FITC (NHS,

Pierce Immunotechnology, Rockford, IL) ester linkage according to the following protocol. Five parts of NHS-FITC were dissolved in *N*-methylpyrrolidone with one part of peptide bound to solid support that still had side chain groups protected, leaving only the N terminus free, thus ensuring that only one FITC group can react per peptide molecule. Two parts of DIEA (diisopropylethylamine) were subsequently added. After 4 to 8 h, the reaction mixture was filtered through a medium fritted glass funnel; cleaved from supporting resin and protecting groups using a cleavage mixture (Applied Biosystems) of crystalline phenol, 1,2-ethanedithiol, thioanisole, deionized H_2O, and trifluoroacetic acid; and purified on a Sephadex G-25 column (Pharmacia Biotech, Quebec, Canada), and the FITC-conjugated peptides were purified by HPLC as previously described (10). Peptides used in this study were dissolved at a concentration of 1 mg/ml in either physiologic saline or medium and filtered through a 0.22-µm pore size filter for sterilization.

K3 and K4, were prepared by the Merrifield solid-phase technique on a Beckman 990C Peptide Synthesizer (Palo Alto, CA) as previously described (14'). The crude preparations were purified by HPLC on a C-18 reverse phase semi-preparative SynChropak RP-P column (synchrom, Linden, IN), using a linear gradient from water to acetonitrile (1.37% acetronitrile/min). For functional assays, peptides were dissolved in saline by adjusting pH to 7.2 with 0.1 N NaOH and were sterilized by filtation through a 0.22 µm filter. Abbreviations used for amino acids: K, lysine; E, glutamic acid; Y tyrosine; A, alanine. Purfied protein derivative (PPD, Statens Seruminstifut Tuberculin Department Copenhagen, Denmark) was used at a concentration of 40µg/ml for a positive control.

Antibodies

mAb-producing hybridomas 10-2.15 (anti-I-Aβ^{s}), MK-D6 (Anti-I-Aβ^{d}), and RA3.3A1 (anti-B220), used for Western blotting and FACS analysis, were purchased from the American Type Culture Collection (Rockville, MD). Horseradish peroxidase-conjugated rabbit and anti-mouse IgG Abs, used for Western blotting, were purchased from Amersham Canada Ltd. (Oakville, Canada).

Cells and hybridomas

TA3 cells were obtained from Dr. L. Glimcher, Harvard Medical School (Boston, MA). As previously described (12). TA3 cell lines exhibit a reduction in surface I-A^{d} expression when passaged in culture over time. In the high expressing clones, I-A^{d} surface expression exceeds I-A^{k}, whereas low expressing clones have greatly reduced I-A^{d} compared to I-A^{k}. For these studies, an intermediate expressing subclone of the TA3 cell line (TA3.11) that has similar levels of I-A^{d} and I-A^{k} expression was used (12).

Immunization and T cell proliferation assay

Mice were immunized in the hind footpads with CLIP peptide (100 µg), K1A2 peptide (100 µg), or OVA (50 µg) emulsified with CFA or IFA (Sigma Chemical Co., St. Louis, MO) using a saline group as a control. After 10 days, popliteal lymph nodes were excised, and a single cell suspension was prepared. Cells from CFA/CLIP-immunized mice and those from control mice were separated as follows. Cell suspensions were incubated for 1 h at 37°C, and the resulting nonadherent supernatant fraction was enriched for T cells on a nylon wool column (13). Adherent macrophages were pooled with column-bound B cells from CFA/CLIP-immunized mice and in turn pooled with T cells enriched from control mice. In other experiments, cell suspensions were used without enrichment. Cells were cultured in 96-well flat-bottom plates (Becton Dickinson Co., Rutherford, NJ) at 2 x 10^{5} cells/well in the presence or the absence of either of both challenge peptides (100 µg/ml for CLIP

peptide, 40 µg/ml of PPD unless otherwise indicated, and 50 µg/ml for OVA) in 200 ml of culture medium (RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Bockneck, Canada), 10 mM HEPES, 2 mM L-glutamine, 5 x 10^{-5} M 2-ME, and 1 U/ml penicillin-streptomycin). After 3 days, cultures were pulsed with 1 µCi/well of [^{3}H]TdR (New England Nuclear-DuPont, Boston, MA) for 16 to 20 h. Incorporation of [^{3}H]TdR was measured using a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). None of the peptides, including CLIP, was toxic to the cells in culture over a wide range of doses, as determined by trypan blue dye exclusion.

In another experiment, BALB/c mice were immunized with 50µg of either K3, K4 or K1A2 peptide along with either saline, CLIP or ovalbumin (323 - 339) peptide emulsified in IFA (Sigma Chemical Co.) in both the hind foot pads. Another group of mice was immunized with 50 µg of ovalbumin protein (Sigma Chemical Co.) along with either saline, CLIP, or K1A2. After ten days, popliteal lymph nodes were removed and single cell suspension was prepared. The cells were then cultured in 96-well flat bottom plates (Becton Dickinson and CO., NJ) at 2x10^5 cells/well in the presence or absence of the peptide (50µg/ml) in 200 µl of culture medium [RPMI 1640, (Gibco, Grand Island, NY) supplemented with 10% FCS (Bockneck, Rexdale, ON, Canada), 10mM HEPES, 2mM L-glutamine, 5x10^5 M 2-mercaptoethanol and 10/ml penicillin/streptomycin]. After 3 days, cultures were pulsed with 1µCi/well of [^{3}H]-thymidine (NEN Du Pont, Boston, MA) for 16-20 h. Incorporation of [^{3}H] thymidine was measured using a liquid scintillation counter (LKB Instruments, Gaithersburg, MD).

FACS analysis

FACS analysis was performed on both TA3 cells and murine lymphocytes. For TA3 cells, 1 x 10^6 cells were incubated with or without CLIP-(85-101) or OVA-(323-339)

peptides at different times and concentrations. Cells were also incubated with OVA or HEL proteins. Cells were then stained for I-A^d and I-A^k surface expression. In other experiment, cells were incubated with FITC-CLIP (100 µg/ml) or FITC-OVA-(323-339) peptide (100 µg/ml) for various times and then analyzed by flow cytometry (12) (Becton Dickinson, CA). Live cells were gated based on propidium iodide exclusion and side/forward laser scatter. Gated cells (20,000e vents/sample) were subsequently analyzed using LYSYS software (Becton Dickinson, Mountain View, CA).

For murine lymphocytes, lymph nodes from CLIP- or non-CLIP-immunized mice (described below) were harvested, and a single cell suspension was prepared. Cells were stained for I-A^d (BALB/c) or I-A^k (C3H) and B220 surface expression and subjected to flow cytometric FACScan analysis as described above.

Western blot analysis

TA3.11 cells (2 x 10^6) were incubated with or without varying concentrations of CLIP peptide (100, 200, and 500 µg/ml) for 24 h at 37°C. Cells were resuspended in lysis buffer (1% Triton X-100, 1% BSA, 1 mM iodoacetamide, 0.2 U/ml aprotinin, 1mM PMSF, 0.01 M Tris, 0.14 M NaCl, and 0.025% NaN_3, pH 8.0) and incubated for 1 h at 4°C. Lysate was cleared by microcentrifugation (10,000 x g. 30 min) and added to an equal volume of 2 x SDS-sample buffer (25% 4 x Tris-SDS (pH 6.8; 6% Tris and 0.4 g of SDS), 20% glycerol, 4% SDS, and 2% 2-ME). Samples were either boiled for 5 min or not boiled and analyzed by electrophoresis by 10% SDS-PAGE. Gels were blotted onto Immobilon paper (Millipore Corp., Bedford, MA) and incubated with primary Ab MK-D6 (anti-I-$A\beta^d$). Secondary horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Canada) was subsequently added to the washed blot and incubated. Washed blots were developed using the enhanced chemiluminescence method (Amersham Canada) and exposed to x-ray film (Dupont De Nemours Co., Wilmington, DE).

Cytokine assays: To determine the cytokine production, lymph node cells ($2x10^6$) form immunized mic were cultured as described above in 24-well flat bottom plates (Corning Glass Works, Coming, NY) in 2 ml medium in presence or absence of the peptides K3, K4 or K1A2 50µg/ml). Supernatents were collected at different time periods and assayed for different cytokine contents. IL-2 contents were assayed by culturing supernatent wisth CTLL cells for 20 h. Cultures were then pulsed with [^{3}H]-thymidine (1µCi/well) and incorporation of [^{3}H]-thymidine was measured as described above. Recombinant IL-2 (20U/ml) (Collaborative Biomedical Products, Bedford, MA) was used as positive control. IL-4 was assayed using IL-4 dependent CT4.S cells (kindly supplied by Dr. B. Chan, Robarts Research Institute, London, Ont.) CT4.S cells were cultured with supermatant for 30 h. [^{3}H]-thymidine (1µCi/well) was added and cultured for additional 18 h. Incorporation of [^{3}H]-thymidine was assayed as described above. IL-4 containing suplernatant from murine rIL-4 cDNA transfected X63Ag8-653 myeloma cells (X63, kindly suppliced by Dr. B.Chan, Robarts Research Institute, London, Ont.) was used as positive control at 20U/ml (19). Supermatants were also assayed for IFN-γ was used as positive control at 40ng/ml.

Antibody response: Mice were immunized with 30µM K3, K4 or K1A2 in 50µl saline emulsified in 50µl of CFA in one hind footpad. After two weeks second shot of the respective peptide (30µM) in IFA was given intraperitoneally. Blood was collected at different time intervals after second shot and sera collected. The isotype of antibodies generated against K3,K4 and K1A2 were detected LISA assay using isotype specific Goat anti-mouse Ig antibody.

Flow Cytometric Analysis: A20 hybridoma cells were stained for the FACS analysis cells were incubated with or without various concentrations of CLIP or ovalbumin peptide for 24

hours at 37°C in 24 well plates at a concentration of $2x10^5$ cells/well. Cells were then washed and incubated with MKD.6 (anti -A^d) antibodies. These cells were then washed and stained with flourescein isothiocynate (FITC)-conjugated goat anti-mouse IgGFc (Jackson Laboratories, Bar Harbor, ME). For negative control, cells were stained with only FITC-conjugated secondary antibody.

Example 2

Effect of incubation of TA3 cells with CLIP peptide or immunogenic peptides and proteins

TA3 cells, were incubated with various concentrations of CLIP peptide-(85-101), OVA peptide-(323-339), HEL, or OVA for various lengths of times (15 min to 24 h) at 37°C. Cells were then stained for the surface expression of $I-A^d$ or $I-A^k$ and analyzed by flow cytometry (Fig. 2, A and B). Surface $I-A^d$ was significantly decreased in a dose-dependent fashion, while the decrease in $I-A^k$ was less pronounced (Fig. 1A). Maximal down-regulation was observed after 24 h using 100 ug/ml of peptide. Previous work has demonstrated that the $A\alpha^kA\beta^d$ heterodimer has increased affinity for CLIP over $A\alpha^kA\beta^k$ heterodimers (7, 14) based on competition assays, and this is a likely explanation for the observed difference in MHC class II surface down-regulation between phenotypes.

Figure 2B demonstrates the effect of incubating the same cells used above with immunogenic Ags. The $I-A^d$-restricted Ag OVA resulted in an increase in surface $I-A^d$, whereas the $I-A^k$-restricted Ag HEL did not. Using a peptide fragment (323-339) of OVA also resulted in a similar increase in surface $I-A^d$ (data not shown). These results are significant because they suggest that 1) CLIP peptide decreases the surface expression of class II expansion in a promiscuous fashion and 2) immunogenic Ags up-regulate class II expression in a class II MHC-restricted fashion, thus confirming previous studies with TA3 cells (12).

Example 3

Incubation of TA3 cells with CLIP peptide increases the intracellular floppy state of I-A^d complexes

It has previously been shown (15) that the unstable state of class II molecules is associated with α/β/Li complexes that migrate slower on SDS-PAGE than molecules in the stable state, which is a reflection of stable binding of the α/β heterodimer with peptide. It has also been reported that binding of CLIP peptides to MHC class II molecules results in the inability of α/β complexes to achieve the compact state (7). To determine whether exogenously added CLIP peptide has an effect on the stability of α/β hetereodimers, TA3.11 cells were cultured with varying amounts of the CLIP peptide (Fig. 3). A dose-dependent increase in the ratio of unstable state of I-A^d molecules (nonboiled) was observed compared with total I-A^d (boiled) of cell lysates, and a point of saturation occurs at a CLIP concentration of 200 μg/ml.

Example 4

Incubation of TA3 cells with CLIP demonstrates the exogenous CLIP does not bind to class II molecules on the cell surface

To determine whether exogenous CLIP was binding directly to cell surface class II MHC or was associating internally, TA3 cells were incubated in the presence of FITC-CLIP or FITC-OVA-(323-339), as a control, for various time periods. Cells were subsequently analyzed by FACS analysis. Figure 4 (A and B) shows that the immunogenic peptide OVA-(323-339) bound to TA3 cell surface Ags to a much greater extent than CLIP, despite the promiscuity of CLIP for binding to class II molecules. This demonstrates that immunogenic peptides are more capable than CLIP of binding to surface class II MHC. Further, time course studies show that CLIP binding appears to plateau at 3 h,

whereas binding of OVA peptide continues to increase with time over a 24-h period.

Example 5

Immunization of Ags with CLIP peptide inhibits in vitro T cell recall response

The inventor investigated what effect(s) CLIP may have in vivo by immunizing BALB/c mice and C3H/HeJ mice with CLIP peptide (100 mg/footpad) in CFA or IFA and, in another experiment, with OVA (50 µg/footpad). Harvested popliteal lymph node cells were challenged in vitro with the priming Ag, PPD, CLIP, or OVA, and the T cell response was measured. Figure 4(A and B) shows the effect of immunizing with CLIP peptide in concert with CFA. BALB/c mice immunized (Fig. 5a) with CLIP and CFA exhibited a significant decrease in T cell response to PPD over that of mice immunized with CFA alone. Similarly, in C3H/HeJ mice, (Fig. 5B), immunization with CFA and CLIP produced a decrease in the in vitro T cell proliferative response to PPD over that of mice immunized with CFA alone.

To determine whether the decreased response was due solely to competition between CFA and CLIP peptide, BALB/c mice were also immunized with CAFA and an unrelated I-A^d-restricted synthetic peptide, K1A2 (EYKEYAAYA(EYA)$_2$) (10, 11). Mice immunized with CFA/saline were used as controls. The T cell response was measured as described above (Fig. 5A), and no decrease in the PPD or K1A2 response was observed, suggesting that CLIP interfered with the PPD while K1A2 has no such effect.

To determine whether CLIP exerts its effects on APCs or T cells, cell mixing experiments were performed using T cells from CFA/saline-immunized or CLIP/CFA-immunized mice. The results of this experiment are shown in Figure 5C. When T cells from control CFA/saline-immunized mice were mixed with APCs from CLIP/CFA-immunized mice, the T cell response was reduced to a similar level as that observed when both APCs and T cells were derived from CLIP/CFA-

immunized mice. When APCs from the CFA/saline-immunized group were mixed with T cells from CLIP/CFA-immunized mice, a T cell response similar to that of the CFA/CLIP group was observed. We believe that this is the result of a lack of priming of T cells in the presence of CLIP, and this lack of priming subsequently yields fewer activated T cells in vitro.

To evaluate the magnitude of the down-regulatory effect of CLIP to PPD, dose-response studies were performed. PPD was used at concentrations of 100, 10, 1, and 0.1 μg/ml. Figure 5D shows that there was a consistently impeded recall response to PPD in the CFA/CLIP-immunized group compared with that in the group immunized with CFA alone.

Finally, to exclude the possibility of the down-regulatory effect being specific to PPD, OVA was used as a priming Ag with CFA and CLIP. As with PPD, the OVA recall response was also reduced when CLIP was co-immunized with OVA (Fig. 4E).

Example 6

Immunization with CLIP peptide decreases surface expression of class II MHC

To determine whether the class II MHC down-regulation observed with CLIP in vitro could be occurring in vivo, BALB/c mice were immunized as described above, and the cells from lymph nodes were stained either for I-A^d surface expression or for B220 (CD45) as a control. Figure 6 (A and B) shows the results of FACS analysis on B lymphocytes and macrophages obtained from the lymph nodes of BALB/c mice. I-A^d surface expression was significantly reduced in the CLIP-immunized group compared with that in the saline control group (Fig. 6B), whereas B220 expression was not (Fig. 6A). Similar results were obtained in C3H/HeJ-derived (I-A^k) lymph node cells (data not shown). These results suggest that the functional results obtained above

may be explained by the lower level of class II expression in CLIP-immunized mice.

Example 7

Effect of CLIP on the cell surface expression of MHC class II : A20 hybridoma cells were incubated with various concentrations of CLIP(85-101) or ovalbumin peptide (323 - 339) for 24 hours. Cells were stained for I-A^d expression and analyzed by flow cytometry. The data presented in Figure 7 show that CLIP down regulated the surface expression of MHC class II on A20 cells in a dose dependent manner. The immunogenic peptide, ovalbumin, on the other hand, up regulated the level of class II expression. These results suggest that addition of exogenous CLIP interferes with its efficient removal from the MHC molecules by H-2M and thus, affects the transport of MHC to the cell surface, where as, the immunogenic peptides bind to the MHC and thus up regulate class II expression.

Example 8

Immunization with CLIP inhibits the In Vivo response to peptide: As the previous results suggested CLIP down regulates the Cell surface expression of mHC, further experiments were done to study its effect on the antigen presentation *in vivo*. Mice were immunized with the CLIP (50 µg/footpad) along with peptide antigens K3, K4, or K1A2 (50 µg/footpad). Peptide K3 has low affinity for MHC while K1A2 has high affinity. K4 has an intermediate affinity for MHC (8'). Ovalbumin peptide (323-339) was used as a control in place of CLIP. Ten days later, lymph nodes were harvested and T-cell proliferation was assayed. Mice immunized with the peptides along with CLIP showed a significant reduction in the proliferative response over mice immunized with peptides alone (Figure 8). However, the magnitude of the down regulation of the response depended on the affinity of the peptide for the MHC. The inhibition of response to the high affinity peptide K1A2

was to a lesser extent than K4 (intermediate affinity) and K3 (low affinity). This suggests that exogenously added CLIP competes with the peptide antigens for binding to MHC and thus, inhibits the presentation of the antigens to T cells resulting in down regulation of the response.

Example 9

Immunization with CLIP shifts the response toward TH2: We have reported earlier that high affinity peptide K1A2 induces the generation of TH1 type response while low affinity peptide K3 induces TH2 type of response. The peptide K4 with intermediate affinity induces both TH1 and TH2 type of responses (8'). To analyze whether down regulation of antigen presentation by CLIP alters the differentiation of TH1 and TH2 cells in response to peptide antigens, mice were immunized with peptides in presence of CLIP and draining lymph nodes were harvested after ten days. Cells were then cultured in presence of peptides, supernatants were collected and assayed for the presence of IL-4 and IFN-γ. The data presented in Figure 9 shows that immunization with peptide in presence of CLIP resulted in a decrease in IFN-γ production by K4 and K1A2 primed cells. On the other hand, IL-4 production by K3 and K4 was up regulated and a little amount of IL-4 was detected in K1A2 primed cell culture. These results suggest that immunization with CLIP along with peptide antigens shifts the response toward TH2 type.

Example 10

Immunization with CLIP shifts the isotype of peptide specific antibodies from IgG2a to IgG1: Mice were immunized with either K3, K4 or K1A2 peptides emulsified in CFA. Two weeks later mice were given a second injection of peptides emulsified in IFA. Mice were bled after two weeks of second injection and serum was separated. The serum was then assayed for the presence of IgG1, IgG2a antibodies. The peptide K3 induces antibodies of IgG1 isotype with very low levels of IgG2a isotype antibodies. Immunization with

CLIP along with the peptide increased the levels of IgG1 antibodies indicating an increase in the Th2 response. On the other hand, immunization with K4 and CLIP did not seem to have an effect on IgG1 response but down regulated the IgG2a response suggesting down regulation of TH1 type of response while TH2 response is maintained. The third peptide, K1A2, displayed the biggest shift in antibody response upon addition of CLIP. The IgG1 antibody response increased significantly when CLIP was added, where as, the IgG2a response was significantly down regulated suggesting a shift from a strong TH1 to a TH2 response (Figure 10). These results confirm the results obtained from cytokine studies.

Example 11

Immunization with CLIP down regulates the Proliferative Response to Ovalbumin Protein: In order to extend the results observed for peptide antigens, CLIP peptide was also used in conjunction with protein antigen. As protein antigens require processing before they are able to bind to MHC it was of interest to determine whether there would be an observable difference between them and peptide antigens. Mice were immunized with ovalbumin protein with or without CLIP in boch the hind footpads. After 10 days, lymph nodes were harvested and a T-cell prolifeation assay was set up. The results presented in Figure 11 show that addition of CLIP down regulated the prlilferative response of mice to ovalbumin protein. Possibly, exogenously added CLIP interferes with the binding of peptide fragments of ovalbumin to MHC molecules thus, decreasing the presentation to T cells resulting in down regulation of proliferative response.

Further, effect of immunization with CLIP on the generation of TH1 and TH2 response by ovalbumin was tested. The results presented in figure 6 show a higher level of Il-4 secretion upon immunization with CLIP and a decreased level of IFN-γ suggesting a shift from TH1 to TH2 type of

response. These results suggest that CLIP can inhibit the response to both peptide and protein antigens.

Example 12

Effect of increasing doses of CLIP on the antigen presentation: To find out the effect of increasing immunization doses of CLIP on the antigen presentation *in vivo* mice were immunized with various concentrations of CLIP in CFA. After 10 days, lymph nodes were removed and proliferation was assayed in response to recall antigen PPD. Our results show that CLIP down regulated the proliferated response in a dose dependent manner. The effect of CLIP reached its maximum at a concentration of 10 µg/ml. Inhibitory effect of CLIP at 100 µg/ml was not much different than at 10 µg/ml.

Example 13

A group of NOL mice, an accepted animal model of autoimmune or Type I diabetes, are treated with an immunogenic composition of CLIP peptide (10 to 50 µg/mouse) and incomplete Freund's adjuvant at around 4 to 8 weeks of age, one treatment per week for 4 weeks.

A control group of NOD mice are treated with adjuvant alone. Development of diabetes is followed by testing for urine glucose and, when urine glucose is detected, by determining blood glucose. Urine and blood glucose are measured by conventional methods.

Immunization with CLIP peptide prevents or delays the development of diabetes in the test group of NOD mice, compared to the control group.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: CLIP IMMUNOMODULATORY PEPTIDE

(iii) NUMBER OF SEQUENCES:4

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(A) COMPUTER: IBM PC compatible
(B) OPERATING SYSTEM: PC-DOS/MS-DOS
(C) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE: 16-MAY-1997
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) PATENT AGENT INFORMATION
(A) NAME: Patricia A. Rae (Dr.)
(B) REFERENCE NUMBER: 6310-12/PAR

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Pro Val Ser Glu Met Arg Met Met Ala Thr Pro Leu Leu Met Arg
1 5 10 15

Pro Met

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: peptide

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Leu Pro Lys Pro Pro Lys Pro Val Ser Lys Met Arg Met Ala Thr Pro
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Leu Leu Met Gln Ala Leu Pro Met
20

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Lys Tyr Lys Glu Tyr Ala Ala Tyr Ala Glu Tyr Ala Glu Tyr Ala
1 5 10 15

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Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn Glu Ala Gly
1 5 10 15

Arg

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of treating or preventing an immune disorder comprising administering to a mammal in need of treatment or susceptible to said disorder a therapeutically or prophylactically effective amount of an MHC class II-associated invariant chain protein or an active fragment or analogue thereof.

2. The method of claim 1 wherein the MHC class II-associated invariant chain protein or effective fragment or analogue thereof is the peptide of Sequence ID NO:1 or Sequence ID NO:2 or an active fragment or analogue thereof.

3. The method of claim 1 wherein the MHC class II-associated invariant chain protein or active fragment or analogue thereof is the peptide of Sequence ID NO:1 or Sequence ID NO:2.

4. The method of claim 2 wherein the peptide is administered as a peptide solution.

5. The method of claim 2 wherein the peptide is administered as an immunogenic composition.

6. The method of claim 2 wherein the mammal is a human.

7. The method of claim 6 wherein the disorder is an autoimmune disorder.

8. The method of claim 7 wherein the autoimmune disorder is selected from the group consisting of autoimmune diabetes, arthritis, multiple sclerosis and systemic lupus erythematosus.

9. The method of claim 6 wherein the disorder is transplantation rejection.

10. A method of reducing the surface expression of MHC class II molecules on antigen presenting cells in a mammal comprising administering to the mammal an MHC class II molecule surface expression reducing effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof.

11. The method of claim 10 wherein the MHC class II-associated invariant chain protein or effective fragment or analogue thereof is the peptide of Sequence ID NO:1 or Sequence ID NO:2 or an effective fragment or analogue thereof.

12. A method of increasing the level of TH2 cells in a mammal comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

13. The method of claim 12 wherein the MHC class II-associated invariant chain protein or effective fragment or analogue thereof is the peptide of Sequence ID NO:1 or Sequence ID NO:2 or an effective fragment or analogue thereof.

14. A method of decreasing the level of TH1 cells in a mammal comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

15. A method of preventing or reducing an immune response in a mammal comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

16. A method of reducing or preventing a T cell response to an antigen in a mammal comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

17. A method of reducing or preventing antigen presentation by antigen presenting cells in a mammal comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

18. A method of treating or preventing, in a mammal, a disorder associated with a T cell response to an antigen comprising administering to the mammal an effective amount of an MHC class II-associated invariant chain protein or an effective fragment or analogue thereof as an immunogenic composition.

19. A pharmaceutical composition comprising an active ingredient selected from the group consisting of :

(a) an isolated nucleic acid sequence encoding an MHC class II-associated invariant chain protein;

(b) an isolated nucleic acid sequence encoding the peptide of Sequence ID NO: 1 or 2;

(c) a substantially pure MHC class II-associated invariant chain protein;

(d) a substantially pure peptide of Sequence ID NO: 1 or 2; and

(e) an active fragment or analogue of any of (a) to (d) and a pharmaceutically acceptable carrier.

20. The composition of claim 19 wherein the carrier is an adjuvant.

21. The composition of claim 19 formulated as an immunogenic composition.

FIGURE 1

Mouse CLIP KPVSQMRMATPLLMRPM

Human CLIP LPKPPKPVSKMRMATPLLMQALPM

Sequence homology between mouse and human CLIP:

```
          85                                    101
          K P V S Q M R M A T P L L M R P M
          | | | |   | | | | | | | | |
81        | | | |   | | | | | | | | |       104
L P K P P K P V S K M R M A T P L L M Q A L P M
```

FIGURE 2

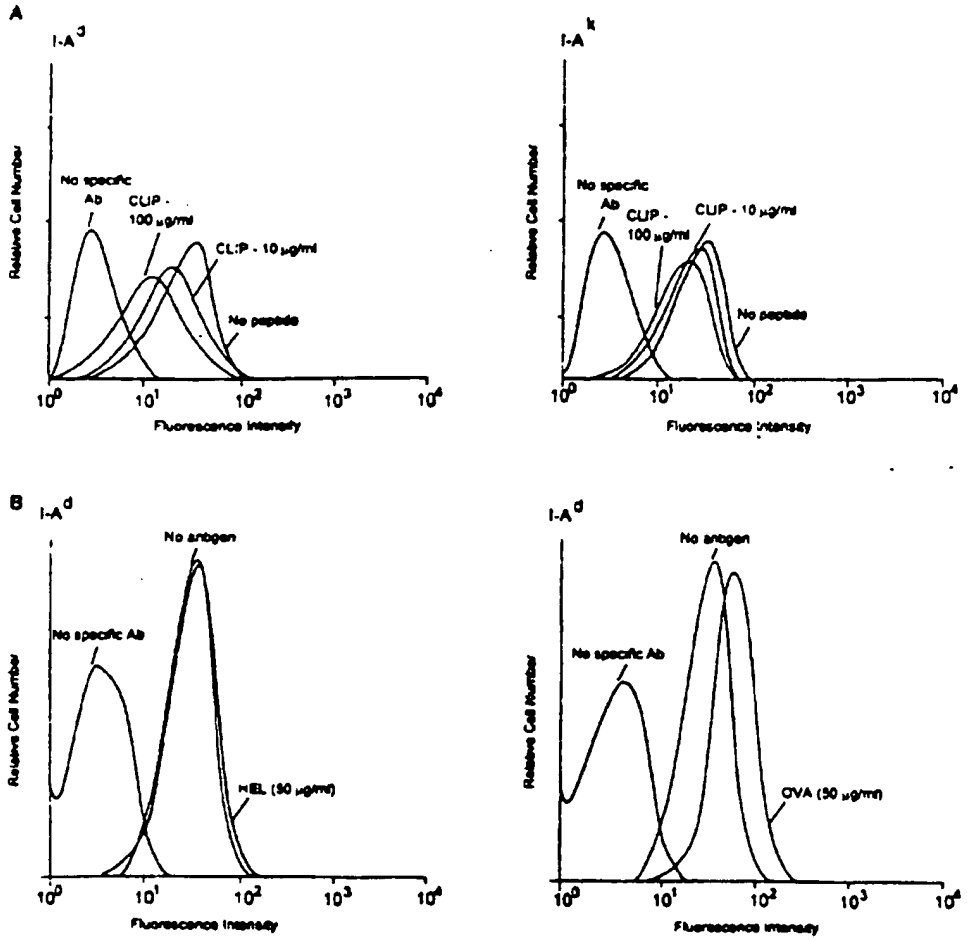

A
I-A^d
No specific Ab
CLIP - 100 µg/ml
CLIP - 10 µg/ml
No peptide
Relative Cell Number
Fluorescence Intensity
I-A^k
No specific Ab
CLIP - 100 µg/ml
CLIP - 10 µg/ml
No peptide
Relative Cell Number
Fluorescence Intensity
B
I-A^d
No antigen
No specific Ab
HEL (50 µg/ml)
Relative Cell Number
Fluorescence Intensity
I-A^d
No antigen
No specific Ab
OVA (50 µg/ml)
Relative Cell Number
Fluorescence Intensity

FIGURE 3

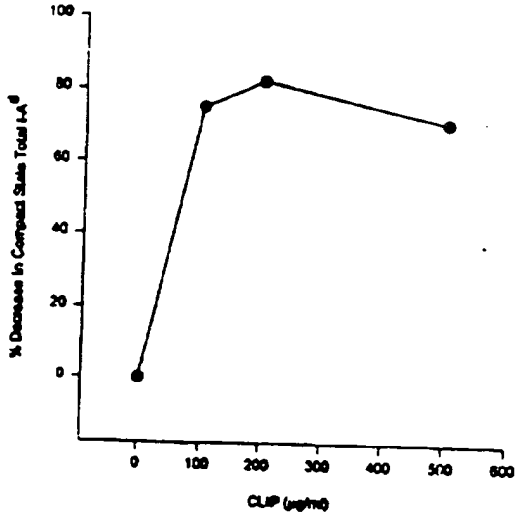


FIGURE 4

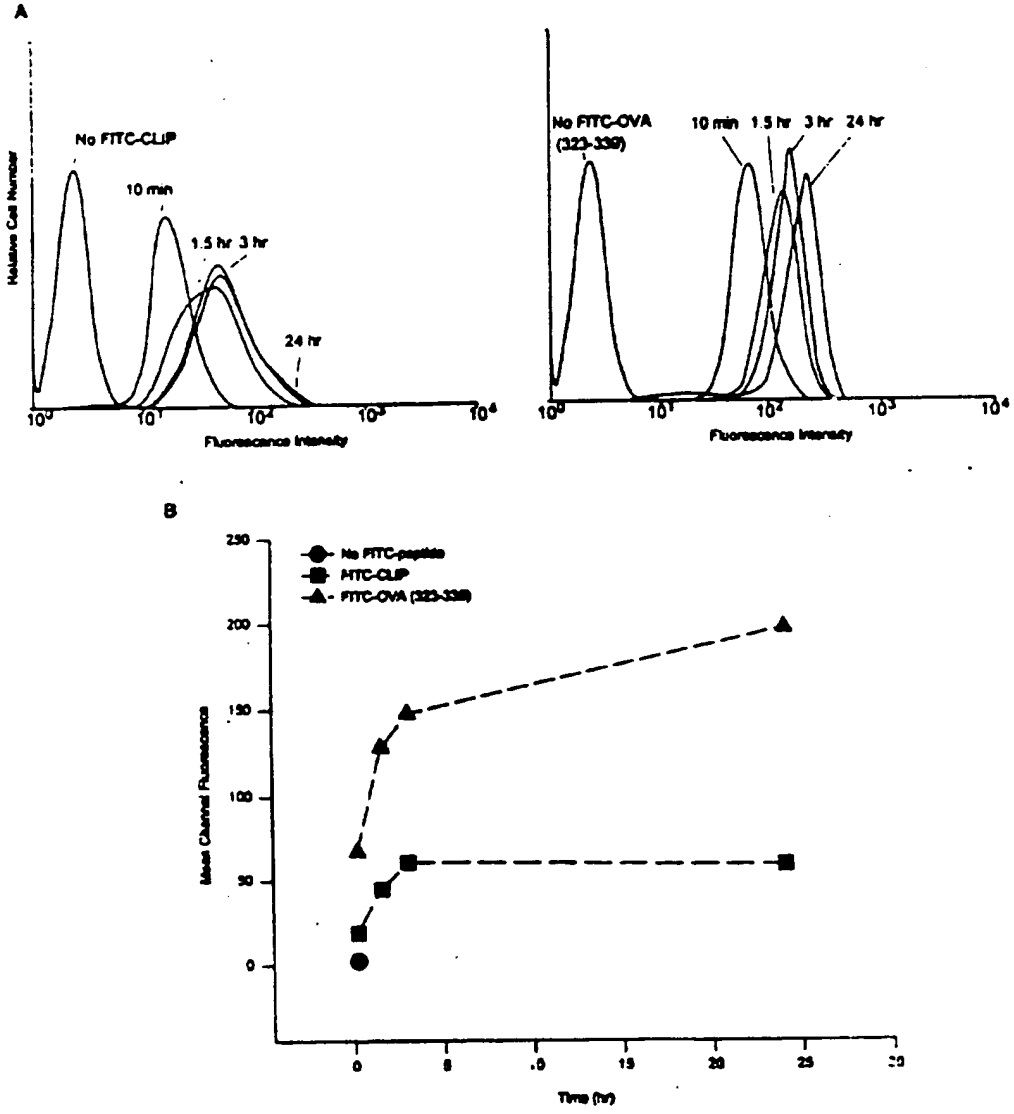

A
No FITC-CLIP
10 min
1.5 hr 3 hr
24 hr
Relative Cell Number
Fluorescence Intensity
No FITC-OVA
(323-339)
10 min 1.5 hr 3 hr 24 hr
Fluorescence Intensity
B
No FITC-peptide
FITC-CLIP
FITC-OVA (323-339)
Mean Channel Fluorescence
Time (hr)

FIGURE 5

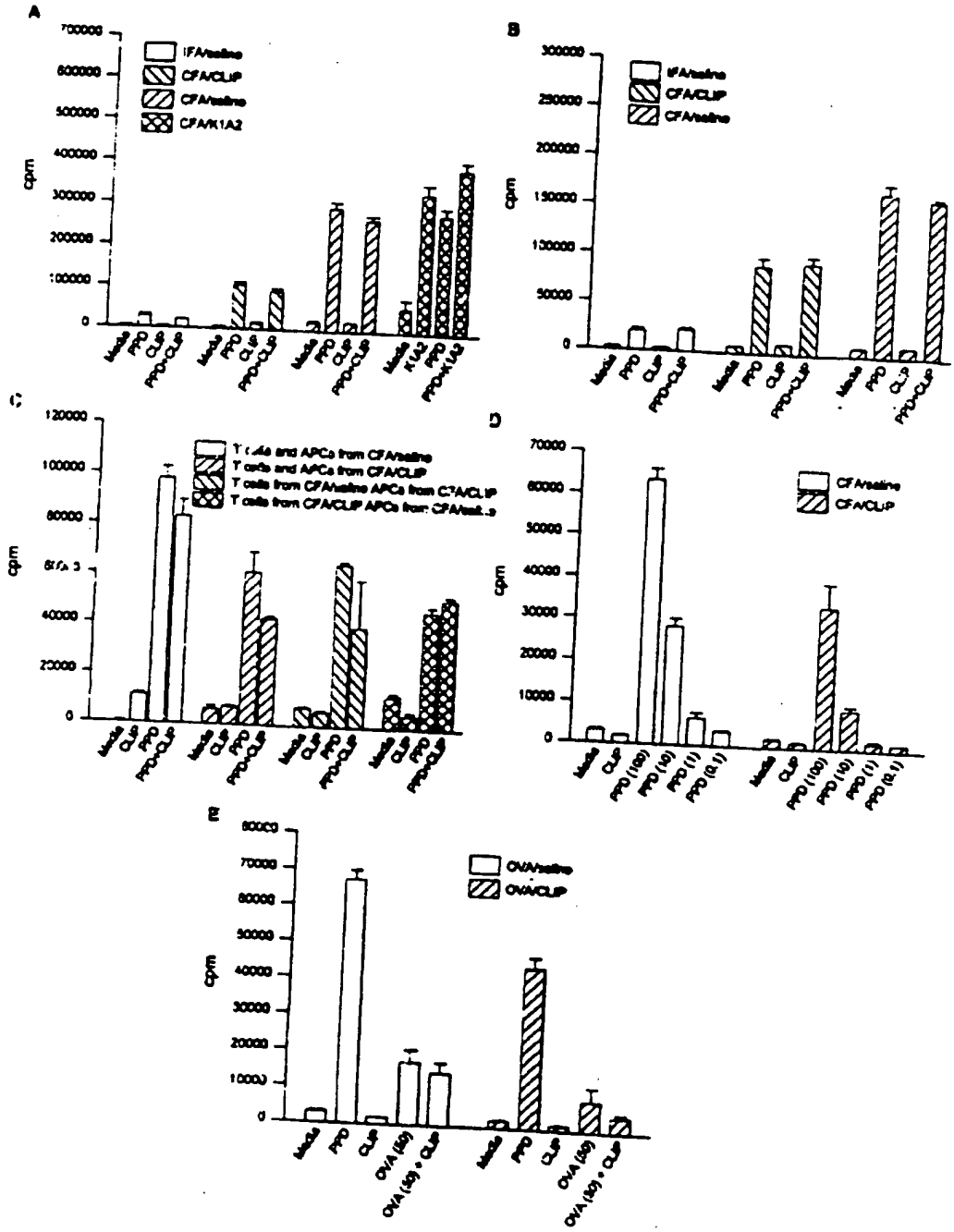

A
B
C
D
E
cpm
IFA/saline
CFA/CLIP
CFA/saline
CFA/K1A2
Media
PPD
CLIP
PPD+CLIP
K1A2
PPD+K1A2
OVA/saline
OVA/CLIP
OVA (50)
OVA (50) + CLIP
PPD (100)
PPD (10)
PPD (1)
PPD (0.1)

FIGURE 6

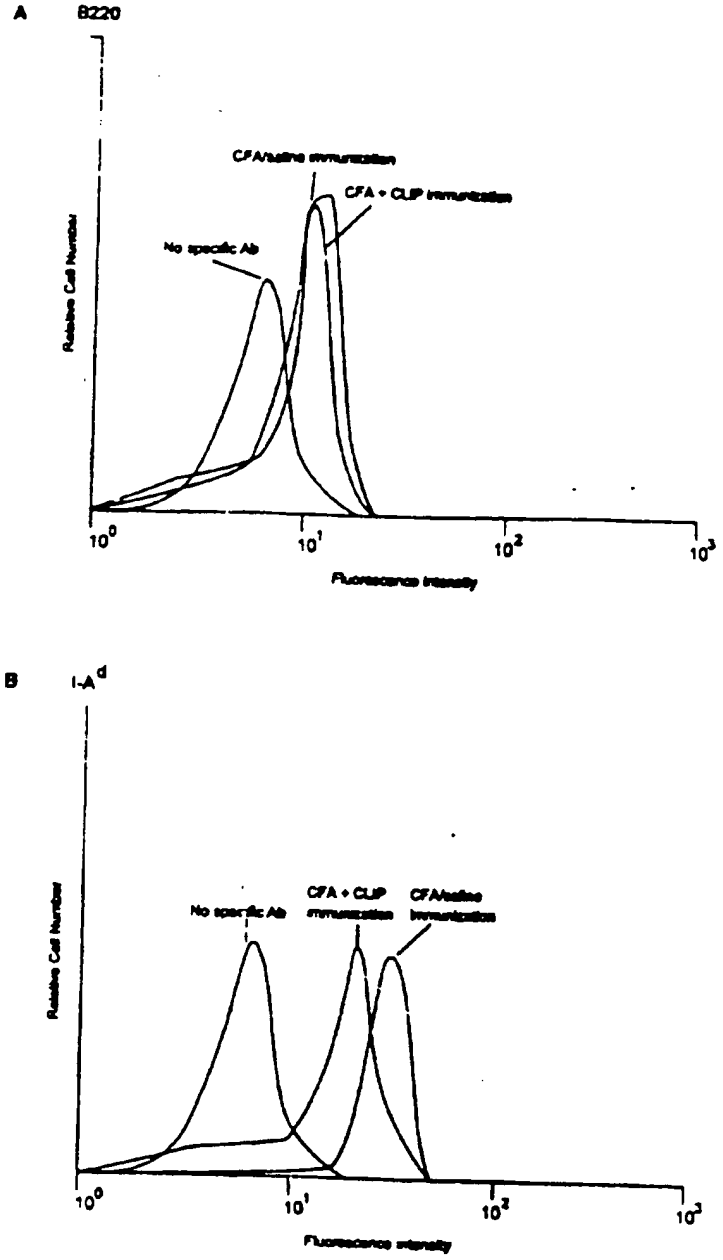

A
B220
CFA/saline immunization
CFA + CLIP immunization
No specific Ab
Relative Cell Number
10^0
10^1
10^2
10^3
Fluorescence Intensity
B
I-A^d
No specific Ab
CFA + CLIP immunization
CFA/saline immunization
Relative Cell Number
10^0
10^1
10^2
10^3
Fluorescence Intensity

FIGURE 7

mmunization with CLIP Downregulates the Proliferative Response to Peptide Antigens

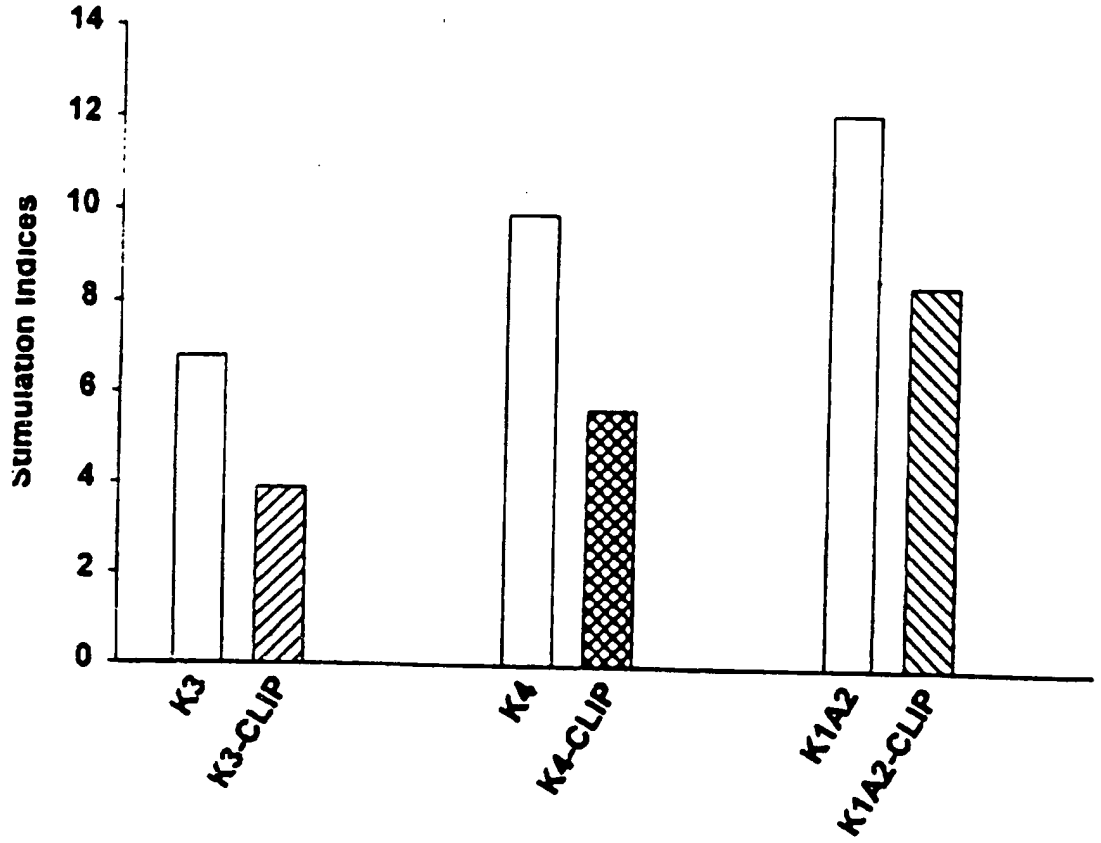


FIGURE 8

Effect of Immunization with CLIP on the Generation of TH1 and TH2 Cells

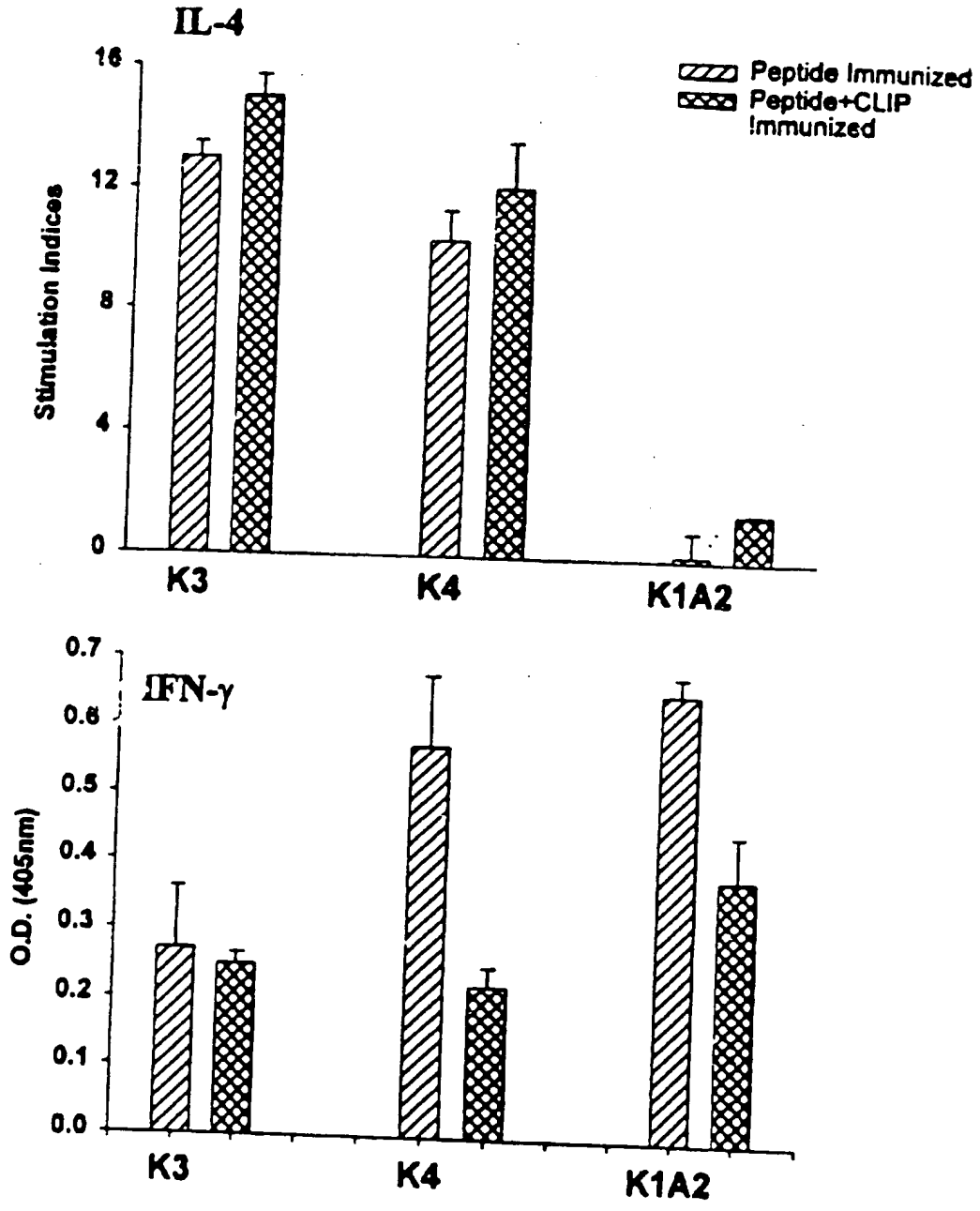


FIGURE 9

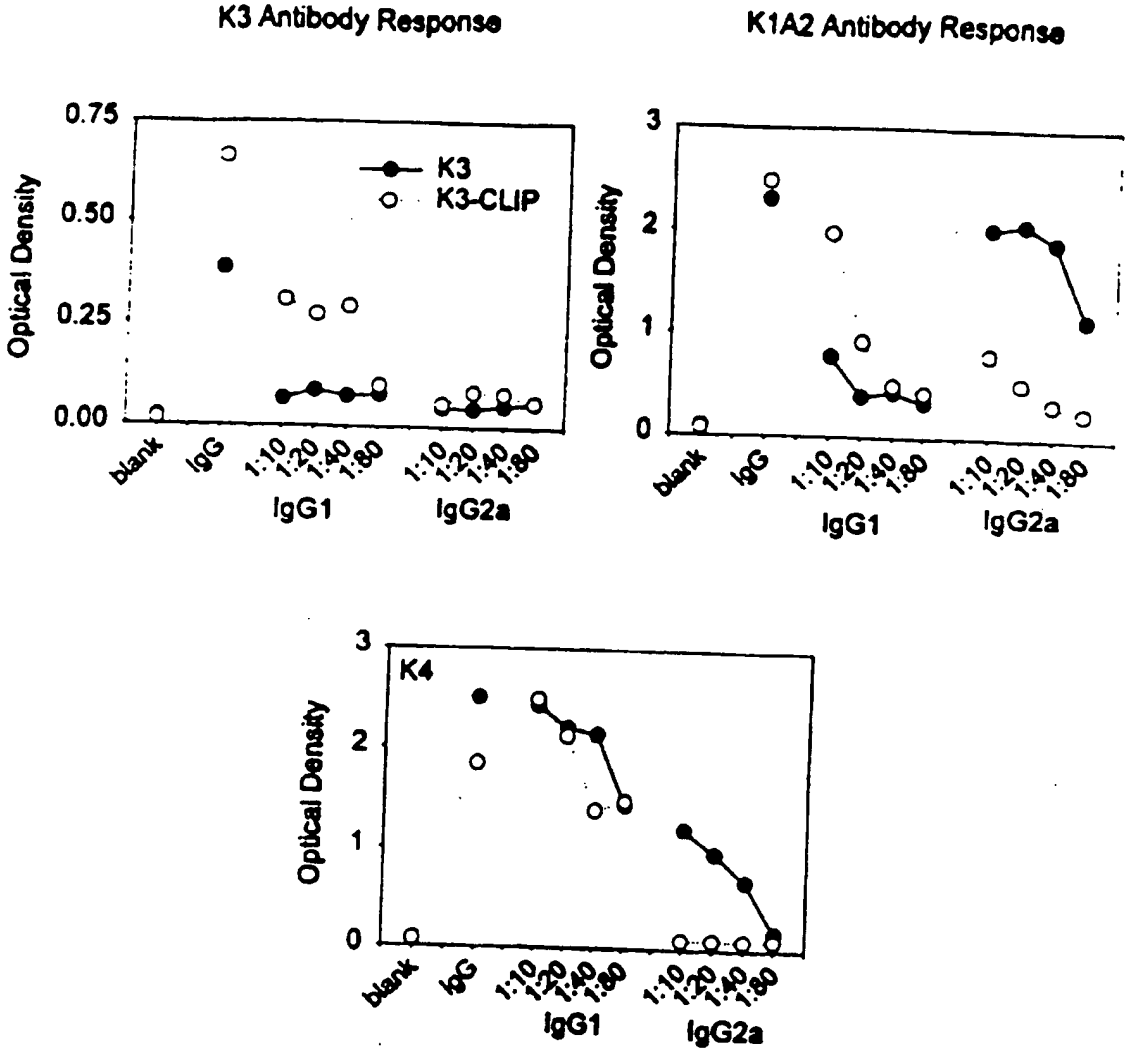

K3 Antibody Response
K1A2 Antibody Response
Optical Density
0.75
0.50
0.25
0.00
K3
K3-CLIP
blank
IgG
1:10
1:20
1:40
1:80
IgG1
IgG2a
3
2
1
0
K4

FIGURE 10

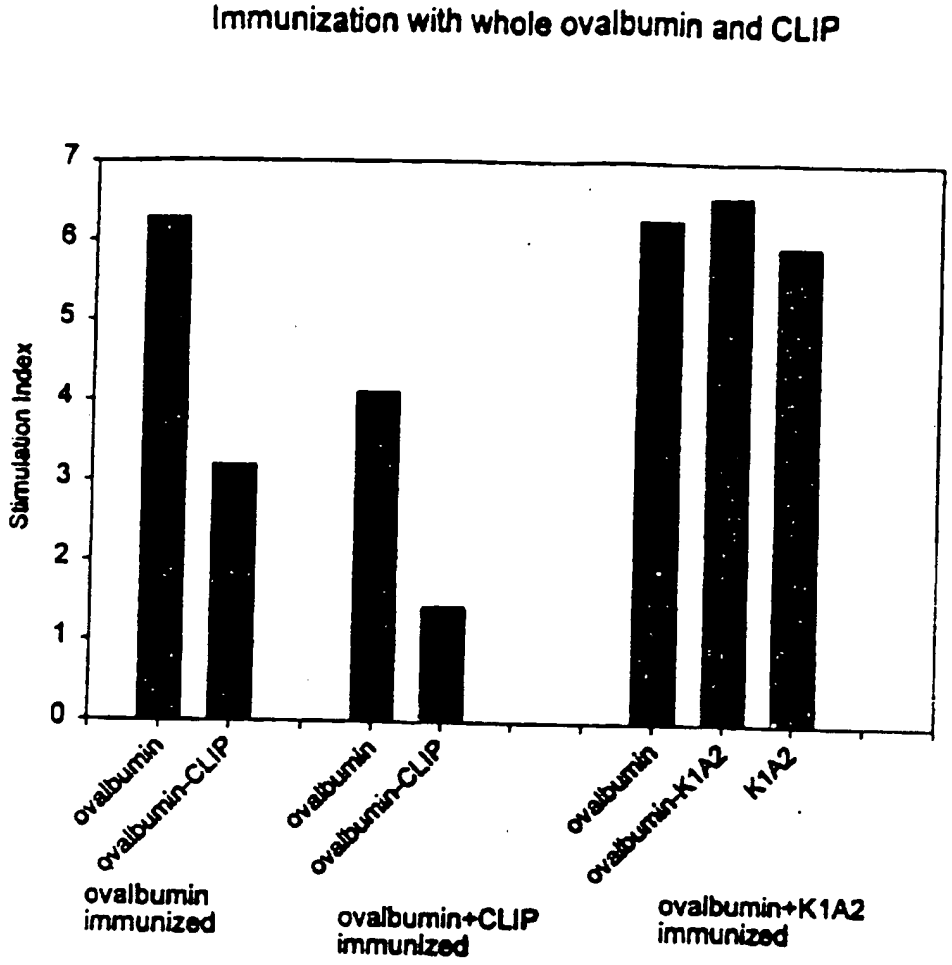
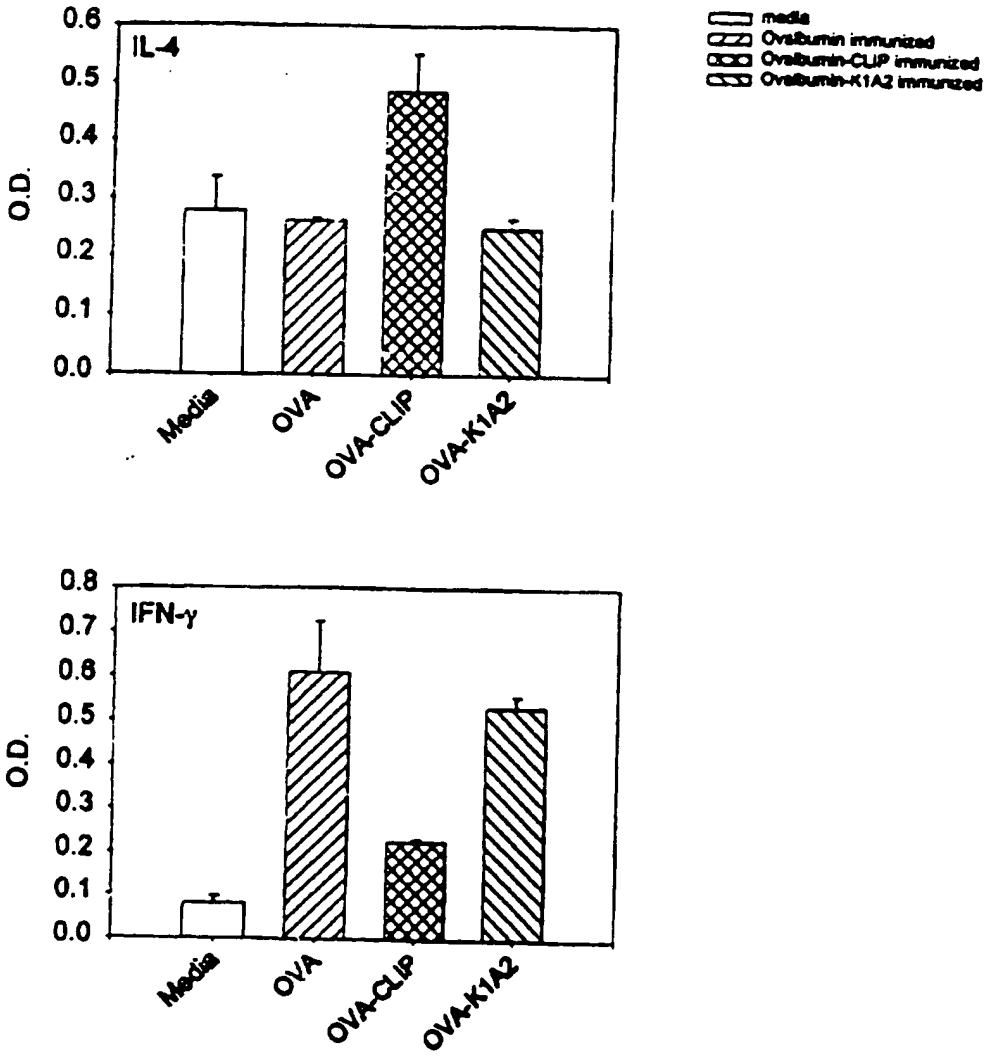

Immunization with whole ovalbumin and CLIP
Stimulation Index
0
1
2
3
4
5
6
7
ovalbumin
ovalbumin-CLIP
ovalbumin
ovalbumin-CLIP
ovalbumin
ovalbumin-K1A2
K1A2
ovalbumin immunized
ovalbumin+CLIP immunized
ovalbumin+K1A2 immunized

FIGURE 11

Effect of CLIP on the Generation of TH1/TH2 Cells in Response to Ovalbumin